AD_____

Award Number: DAMD17-96-1-6067

TITLE: The Role of the Novel Nuclear Tyrosine Kinase, RAK, in Breast Cancer Biology

PRINCIPAL INVESTIGATOR: Paul Armistead, Ph.D.
Holden Thorp, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill, North Carolina 27599-1350

REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jul 96 - 30 Jun 00)

4. TITLE AND SUBTITLE
The Role of the Novel Nuclear Tyrosine Kinase, RAK, in Breast Cancer Biology

5. FUNDING NUMBERS
DAMD17-96-1-6067

6. AUTHOR(S)
Paul Armistead, Ph.D.
Holden Thorp, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina
Chapel Hill, North Carolina 27599-1350

E-MAIL:
armistp@med.unc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The research supported by this grant is intended to investigate the feasibility of an electrocatalytic guanine oxidation reaction as an mRNA quantification method. This system is to be tested first by quantifying the mRNA of Rak nuclear tyrosine kinase in both cell culture and breast tissue samples. To accomplish this goal a competitive RT-PCR assay was designed to measure the absolute quantity of Rak mRNA in each sample. This absolute quantity was compared to the electrochemical signal generated by oxidation of the Rak RT-PCR products immobilized to the indium tin oxide (ITO) electrodes used in this study. This final report presents data that show Rak to be overexpressed in 33% of the breast cancers analyzed. Rak mRNA in normal breast tissue is expressed at the level of roughly 400 zmol/μg total RNA; however, Rak was overexpressed to the level of roughly 2 amol/μg total RNA in some breast tumors analyzed. The breast tumors that exhibited this high level of overexpression by competitive RT-PCR also exhibited the largest electrochemical signal (current) when catalytic guanine oxidation was used as the detection and quantification method.

14. SUBJECT TERMS
Breast Cancer, mRNA quantification, electrocatalysis

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

✓ Where copyrighted material is quoted, permission has been obtained to use such material.

✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Paul M. Armistead 7/1/00

PI - Signature Date

Table of Contents

INTRODUCTION

The Rak nuclear tyrosine kinase is overexpressed in roughly 30% of breast malignancies.[1] This kinase associates with the RB tumor suppressor protein, and maybe involved with tumor suppression in breast cancer.[2] To investigate the biological and clinical significance of Rak in greater detail, experiments were performed to design two assays to determine Rak mRNA expression in both cell culture and breast tumors. One of these assays used a traditional competitive RT-PCR approach, while the other used RT-PCR coupled with an electrochemical detection system that is based upon the catalytic oxidation of guanine residues. Upon creation of these bioassays, Rak expression was to be monitored in cell cultures that were microinjected with an epitope of the Rak peptide.

BODY

In this project, traditional biochemical methods as well as electrochemical techniques were used to determine the mRNA expression level of Rak nuclear tyrosine kinase in a series of breast tissue samples. Rak (also called Frk) is a distant relative of the Src family of tyrosine kinases.[1] The coding sequence for Rak is 2,827 bp, and it is roughly 50% identical to Src. Other members of the Src family have roughly 70% identity to Src. The gene encodes a 54,000 Dalton protein that contains a putative Mg^{2+}-ATP binding site and both an SH2 and SH3 domain. Rak also contains a carboxy-terminal tyrosine that can be phosphorylated by CSK.[1] The most striking difference between Rak and the other members of the Src family is that Rak does not contain either a myristylation or palmitylation site, which implies that Rak does not associate to the cell membrane.[1] Rak does contain a putative nuclear localization signal, and cell fractionation studies support the hypothesis that Rak does indeed localize in the nucleus.[1] In the nucleus, Rak has been shown to associate with the tumor suppressor protein RB.[2] The role of Rak in cancer is less well defined than that of Src. Rak is expressed in epithelial but not messenchymally derived tissues. It is overexpressed in roughly 30% of human epithelial (breast and colon) cancers.[1]

Previous work in our laboratory has shown that catalytic cyclic voltammetry can detect trace quantities of DNA. In these experiments, known quantities of DNA were

adsorbed onto indium tin oxide (ITO) electrodes. The electrodes were placed in an electrochemical cell that was filled with a 10 μM solution of the inorganic complex $Ru(bpy)_3^{2+}$.[3] DNA detection was observed through catalytic oxidation of the guanine base according to the following reactions:[4]

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + \text{guanine} \rightarrow Ru(bpy)_3^{2+} + \text{guanine}^+ \quad (2)$$

The resulting cyclic voltammmogram shows a dramatic enhancement in the $Ru(bpy)_3^{2+}$ oxidation wave as the generated $Ru(bpy)_3^{3+}$ is recycled to $Ru(bpy)_3^{2+}$ by electrons from the guanine bases. Experiments with model PCR products showed that the sensitivity of this system was 44 amol/mm^2 of electrode area. Because the electrodes used have an area of 12.6 mm^2, the practical detection limit for this system is roughly 550 amol.[3]

The mRNA of most signaling molecules, including Rak, are present in quantities of 0.1 – 1.0 amol/μg total RNA.[5] This miniscule quantity makes direct electrochemical detection of the specific mRNA impossible at the large electrodes that are available to us at this time. Because of these sensitivity limitations, chemical amplification of the specific Rak mRNA by RT-PCR was used to dramatically increase the sensitivity and specificity of the expression measurements.

Because the electrochemical detection system cannot distinguish between two differently sized products, the use of a co-amplification standard is not possible. As a result, independent competitive RT-PCR measurements were performed on each sample to accurately measure the absolute expression level of Rak mRNA. This competitve RT-PCR assay was previously described in the annual report dated 07/27/99.

EXPERIMENTAL

Materials and Reagents

All oligonucleotides were synthesized at the Lineberger Comprehensive Cancer Center Nucleic Acid Core Facility at the University of North Carolina at Chapel Hill. Tissue Samples were from the Tissue Procurement Facility at UNC-CH. Of the seven tissue samples studied, sample 35A was normal tissue, sample 54B was a primary tumor with no signs of invasion through the basal lamina, and the others were invasive breast

tumors. Sample 35B was an invasive breast tumor from the same patient as sample 35A. BT-474 cells were a gift from the lab of Dr. Bill Cance. All water used was in-house distilled water that was further purified on a Milli-Q water purification system. Water for use in RNA manipulations was further treated with diethyl pyrocarbonate, DEPC, (Sigma, St. Louis, MO). N,N-dimethyl formamide, sodium phosphate, sodium acetate and sodium chloride were from Mallinckrodt (Paris, KY). $Ru(bpy)_3Cl_2$ was purchased from Aldrich (Milwaukee, WI) and purified by recrystallization from acetonitrile. ITO electrodes were purchased from Delta Technologies (Stillwater, MN). Total RNA from both cell culture and tissue samples were purified on Qiagen RNeasy columns according to the manufacturer's directions. All electrochemical measurements were performed in a one-compartment cell on a Princeton Applied Research (Princeton, NJ) Model 273A potentiostat that was controlled by a Pentium computer. An Ag/AgCl reference electrode was used (Cypress Systems, Lawrence, KS), and a platinum wire was used as the auxiliary electrode. Fluorescent staining of DNA in agarose was performed using SYBR-green (Molecular Probes, Eugene, OR). Fluorescently stained gels were scanned on a Molecular Dynamics (San Jose, CA) Storm 860 phosphorimager using the blue fluorescence mode. Scanned gels were analyzed using ImageQuaNT software.

Competitive RT-PCR

Synthesis of an appropriate Rak mRNA competitor followed the strategy of Totze et al.[6] A Rak RT-PCR product was produced that contained the T7 promotor sequence as well as a "loop-out" region. The reverse transcription was carried out as follows. A 14 μL reaction mix consisting of 1 μg total BT-474 RNA, 714 μM dNTP's (Amersham), 0.4 μg/μL random hexamers (Life Technologies) was prepared. The mix was heated to 75°C for 3 min and immediately cooled on ice. To the mix was added 4 μL 5x buffer (Life Technologies), 1μL RNasin (Promega), and 1 μL (200 U) M-MLV reverse transcriptase (Life Technologies). The reaction was allowed to proceed for 1 h at 42°C and then stopped by heating the mix to 90°C for 10 min.

A 50 μL PCR was made that contained 5 μL of the reverse transcription mix in 1x buffer, 2.5 mM $MgCl_2$, 125 μM dNTP's, 250 nM forward primer (TAA TAC GAC TCA CTC TAG GGC CTA TCT GGA GTC TCG GAA GCA GAT TTT GGA CTT GCC

AGA), 250 nM reverse primer (GGG CAC CTG TCA TAC CAC TGT), and 1 U Taq polymerase (Life Technologies). The reaction was performed with 1 cycle of 95°C for 5 min followed by 30 cycles of 94°C for 20 s, 62°C for 30 s, 72°C for 40 s and 1 cycle 72°C for 5 min. The purity of the product was determined by agarose gel electrophoresis. The gel revealed a single band at the appropriate size (250 bp). The PCR product was purified on Qiagen Qiaquick PCR purification columns.

In-vitro transcription was performed by assembling a 20 μL reaction mix that contained 40 mM Tris (pH = 7.5), 10 mM $MgCl_2$, 10 mM DTT, 8 mM NTPs, 50 μg/mL BSA, 170 nM PCR template, and 1x T7 polymerase that was a gift from the lab of Dr. Kevin Weeks. The reaction was allowed to proceed for 7 h at 37°C. Following the reaction, 1 μL DNase (Ambion) were added to the reaction and allowed to incubate for 30 min. To the reaction were added 15 μL of 5 M ammonium acetate, 15 μL 100 mM sodium EDTA, and 100 μL DEPC water. The reaction was extracted with acid phenol/chloroform/isoamyl alcohol. The organic phase was back-extracted with 50 μL DEPC water. The 200 μL aqueous mix was precipitated with ice-cold 2-propanol overnight. The RNA pellet was resuspended in TE buffer and run on a 4% denaturing polyacrylamide gel. The transcription products were visualized by UV shadowing, and the single visible product was cut from the gel using a clean razor blade. The gel fragment was placed into 300 μL of eluting solution containing 500 mM ammonium acetate, 1 mM EDTA, and 0.2% SDS (Ambion). The gel slice was kept in the solution overnight at 4°C. The eluting solution was transferred from the gel slice, and the eluted RNA was ethanol precipitated overnight revealing a glassy pellet. The pellet was resuspended in TE buffer. The amount of product, 1.8 μg (23 pmol), was determined by UV-vis spectroscopy at 260 nm.

Competitive RT-PCR was performed by preparing 6 reverse transcription mixes for each tissue sample. A 15 μL reaction mix was prepared that contained 0.5 μg total RNA, a known quantity of the competitor (either 31.3 zmol, 62.5 zmol, 125 zmol, 250 zmol, 500 zmol, or 1,000 zmol), 667 μM dNTPs and 667 nM reverse primer. The mixes were heated and cooled on ice as described above. To each mix was added 4 μL 5x buffer and 1 μL (200 U) M-MLV reverse transcriptase. The reverse transcription and

PCR were performed as described above except a different forward primer (GGC CTA TCT GGA GTC TCG GAA) was used. Each reaction mix was run on a 2% agarose gel and stained with SYBR-Green for 20 min. Staining with SYBR-Green was performed after the electrophoresis to minimize measurement errors due to dye migration in the gel. The stained gel was scanned with a Molecular Dynamics Storm 860 phosphorimager in the blue fluorescence mode. The band intensities resulting from both the native Rak mRNA amplification (296 bp) and the competitor RNA amplification (232 bp) were measured, and background fluorescence was subtracted. For each tissue sample the logarithm of the competitor signal/native signal in each mix was plotted versus the logarithm of the amount of competitor added for each mix. The best linear fit was calculated, and the antilogarithm of x at y = 0 was calculated[7]. This value yielded the absolute quantity of Rak mRNA in the tissue sample.

Conventional RT-PCR Amplification of Rak

RT-PCR of Rak was performed on all tissue samples in parallel, except sample 55B, which did not yield enough RNA. Six reverse transcription reactions were assembled (1 for each sample). Each 15 μL reaction contained 0.5 μg total RNA, 0.4 μg/μL random hexamers, and 667 μM dNTPs. The random hexamers and dNTPs were originally pooled into a master mix and then pipetted into each individual reverse transcription mix. The mixes were heated and cooled as described above. Four μL of 5x buffer and 1 μL (200 U) M-MLV was added. These reagents were pre-mixed to ensure that equivalent amounts of enzyme were added to each reverse transcription reaction. The reactions were again performed at 42°C for 1 h and were stopped by heating to 90°C for 10 min.

PCR was assembled for each tissue sample as a 100 μL solution that contained 10 μL of reverse transcription mix, 1x buffer, 2.5 mM $MgCl_2$, 125 μM dNTP's, 250 nM forward primer (GGC CTA TCT GGA GTC TCG GAA), 250 nM reverse primer (GGG CAC CTG TCA TAC CAC TGT), and 2 U Taq polymerase. The reagents were premixed except the reverse transcription mix. Ninety μL of the master mix was added to 10 μL of the reverse transcription mix. This step again ensured that equivalent amounts of Taq were added to each reaction. The reaction proceeded as follows: 1 cycle

of 95°C for 5 min followed by 28 cycles of 94°C for 20 s, 62°C for 30s, 72°C for 40s and 1 cycle 72°C for 5 min. Five μL of each product were analyzed by agarose gel electrophoresis.

Purification of Rak RT-PCR Products

After RT-PCR, 90 μL of each PCR were purified on Qiagen Qiaquick PCR purification columns. The purification was performed according to the manufacturer's instructions, except for the final elution step. At this step, each column was treated with 30 μL of 10 mM sodium acetate pH = 8.0 (pH raised by addition of sodium hydroxide). The columns were allowed to sit for one minute and were then centrifuged at 14,000 rpm for 2 min. Again, 5 μL of each product were analyzed by agarose gel electrophoresis. These gels confirmed that the relative amounts of RT-PCR among samples did not change significantly after purification.

Electrochemical Detection of Rak RT-PCR Products

ITO electrodes were cleaned using the protocol of Willit and Bowden.[8] The electrodes were cleaned by sonication in 2-propanol for 15 min, and twice in Milli-Q water each time for 15 min. The electrodes were air-dried. For each electrode, 5 μL of purified PCR product were added to 45 μL DMF. The DMF mixtures were pipetted onto the electrodes, which were placed in a constant humidity chamber for 30 min.[3] The electrodes were then washed in Milli-Q water for 3 min on a rotary mixer and allowed to air dry.

The DNA modified electrodes were placed in a one-compartment electrochemical cell. The cell contained 150 μL of 10 μM $Ru(bpy)_3^{2+}$ in 50 mM sodium phosphate buffer at pH = 7.0. Electrochemical detection was performed using cyclic voltammetry. The potential range was 0-1,300 mV, at a scan rate of 10 V/s.[3] All measurements were made relative to an Ag/AgCl reference electrode. Because of the large charging currents generated at this fast scan rate, background subtraction was performed. The background scans were taken of ITO electrodes that had been treated with a 9:1 DMF:sodium acetate solution and washed as above. The background electrodes were placed in the same electrochemical cell, and cyclic voltammetry was performed only in the presence of 50 mM sodium phosphate.

RESULTS

Absolute Quantification of Rak mRNA

Competitive RT-PCR was performed on several breast tissue samples so that all subsequent experiments could be related back to a well-established quantification method. A typical agarose gel of a competitive RT-PCR and its analysis are shown in Figure 1. Rak competitive RT-PCR was performed on total RNA from seven breast tissue samples as well as total RNA from BT-474 cultured cells. The linear fits from all of the analyses had an average R-value of 0.99 ± 0.01 and an average slope of 0.98 ± 0.14. A slope of unity indicates that the competitor RNA amplifies with the same efficiency as the native RNA.[7] The compiled competitive RT-PCR results are shown in Figure 2.

Comparison of Rak RT-PCR and Competitive RT-PCR

Because our current electrochemical detection scheme can not differentiate between two differently sized DNA fragments in the same mix, conventional RT-PCR was performed on each tissue sample so than only one product would be produced. Figure 3 shows the relative amounts of RT-PCR product produced from each of the 6 breast tissue samples studied. Results from conventional RT-PCR roughly reflect the relative quantities of Rak in each sample. However, the dramatic differences in expression that were revealed in the competitive RT-PCR experiments are diminished in the conventional RT-PCR despite the fact that the conventional RT-PCR was optimized with respect to amount of Taq used and number of cycles performed. From this result, it is apparent that conventional RT-PCR can only be used as a crude indicator of expression.

Relative Amounts of RT-PCR Products after Purification

For the PCR products to be detected electrochemically, they first had to be purified. Purification is necessary because of the large amount of primers and nucleotides in the reaction mixture. These reagents are electrochemically active, and adsorb onto the ITO surface. Purification also accomplished the goal of concentrating the PCR samples. By increasing the concentration of the samples, the time necessary for the DNA to adsorb onto the electrode was reduced. Purification of the PCR products can be

accomplished quickly and rapidly through the use of silica purification columns. In these experiments, the Qiagen Qiaquick PCR purification columns were used with 10 mM sodium acetate (pH = 8.0) as the elution buffer. Figure 4 shows the relative amounts of Rak RT-PCR products after purification. This figure shows that the relative amounts of the RT-PCR products still roughly reflect the trend observed in the competitive RT-PCR experiments. Figure 5 shows a comparison of the relative product intensities from the competitive RT-PCR experiments and the conventional RT-PCR experiments both before and after purification.

Comparison of Electrochemical Signals and Gel Band Intensities

The results above indicate that conventional RT-PCR with subsequent purification can be used to provide a semi-quantitative analysis of Rak mRNA expression. The results are comparable to results from competitive RT-PCR studies although the inter-sample differences are diminished in the conventional RT-PCR experiments. If electrochemical quantification of conventional RT-PCR products correlated with the amount of RT-PCR product revealed by agarose gel electrophoresis, it would therefore be possible to use electrochemical quantification as another semi-quantitative measure of gene expression.

Cyclic voltammetry is an electrochemical technique in which the potential at the working electrode is ramped to some value and then ramped back to is original value. As the potential reaches the redox potential of the analyte, current is transferred and detected by the electrode. Previous work has shown that the amount of current transferred to the electrode is linearly proportional to the amount of DNA adsorbed to the electrode surface.[3] Figure 6 shows representative cyclic voltammograms of ITO electrodes modified with the Rak RT-PCR products from the breast tissue samples. Figure 7 shows a comparison of the Rak mRNA quantification data. The figure shows that there is good agreement between the current measurements and post-purification band intensities and that both methods correlate to the actual mRNA values determined by competitive RT-PCR. While conventional RT-PCR is not as good as competitive RT-PCR for measuring differences in mRNA expression, it is able to give some general information on the expression. This information is retained through both purification and electrochemical detection steps.

MICROINJECTION OF RAK PEPTIDE (TASK 2)

Microinjection experiments were not able to be performed because of technical problems in our laboratory. Our laboratory is not equipped to performed cell-culture experiments, and the laboratory with which we collaborated could not perform the large amounts of microinjection experiments that would be been need to complete Task 2.

RESEARCH ACCOMPLISHMENTS

- A new method for the attachment of DNA onto ITO electrodes has been developed.
  - This method is rapid (<1 h) and involves no coupling reagents.
  - Catalytic electrochemical detection of adsorbed DNA has a sensitivity limit of 44 amol/mm^2.
- Rak mRNA expression has been determined for BT-474 cells and in a series of 7 breast tissue samples.
  - Rak expression in BT-474 cells is 260 ± 70 zmol/μg total RNA.
  - Rak expression in breast tissue samples ranges from 440 ± 85 to 2,280 ± 240 zmol/μg total RNA.
- Rak mRNA expression determined by RT-PCR with electrochemical detection correlates with the analogous competitive RT-PCR experiments.
- Rak is overexpressed (> 2 fold over normal expression (sample 35A)) in roughly 33% of breast tumors.

REPORTABLE OUTCOMES

- Characterization of the sensitivity limits of this electrochemical detection scheme have been accepted as a paper in Analytical Chemistry (in press).
- This electrochemical detection scheme was presented as a poster at the National M.D./Ph.D. Conference in Aspen CO July 16-18, 1999.
- The electrochemical quantification of Rak mRNA in breast tissue samples and in BT-474 cells will be submitted as a paper to a journal soon.
- The electrochemical and absolute quantification of Rak RT-PCR products was presented as a poster at the Department of Defense Era of Hope Conference in Atlanta GA June 8-12, 2000.
- Paul Armistead earned a Ph.D. in Chemistry through this funding.

CONCLUSIONS

The previous annual report dated 07/27/99 described the development of a catalytic electrochemical DNA detection system. This electrochemical system can detect DNA at ITO electrodes with a sensitivity limit of roughly 44 amol/mm^2.[3] Investigations into the physical chemistry of this catalytic reaction indicate that further gains in sensitivity can be achieved by simply reducing the size of the electrode to a micron scale.[9] The technology to perform such a task is available, but not to our laboratory at this time. Because of the limitations of large (12.6 mm^2) ITO electrodes, a gene expression sensor based upon RT-PCR was designed for Rak nuclear tyrosine kinase.[10]

Despite RT-PCR's problems with regard to mRNA quantification, electrochemical quantification of Rak RT-PCR products correlated with more exact measurements of Rak mRNA expression that were determined by competitive RT-PCR. The competitive RT-PCR assay was performed on BT-474 cells and 7 breast tissue samples. Rak expression was lowest in the BT-474 cells (260 ± 70 zmol/μg) followed by the one normal breast tissue sample (35A, 440 ± 85 zmol/μg) analyzed. Overexpression (defined as expression greater than 2-fold greater than expression in sample 35A) was observed in 33% (2 of 6) of the malignant breast tissue samples examined. Both of these samples showed pathologic evidence of invasion through the basement membrane. These overexpression results were roughly mirrored by the RT-PCR experiments with electrochemical detection; however, the inter-sample differences in expression evident by competitive RT-PCR were reduced.[10]

Rak overexpression has been previously observed; however, absolute quantification techniques were not performed in those experiments.[1] The absolute expression experiments performed in this study reveal that Rak overexpression is relatively limited (i.e. overexpression of Rak in malignant breast tissue was only found to be roughly 5x that of Rak expression in normal breast tissue). This is in contrast to genes like Her-2 whose expression can vary over 4 orders of magnitude.[5,11] That electrochemical detection can distinguish such small variations in expression, indicates that a similar electrochemical detection system could be used for other genes that have a greater range of expression.

Expression analysis of diseased tissue will likely become a useful clinical tool as the underlying causes of many diseases including breast cancer are elucidated. The current technology to perform such analyses is typically based upon optical detection of fluorescently labeled nucleotide probes.[12-15] This detection approach is limited by both cost and time considerations. Electrochemical detection based upon guanine oxidation could greatly facilitate expression analysis because the detection equipment would be significantly less expensive, and the time to perform such tasks would be lessened because there would be no chemical labeling step in the sample preparations.

The feasibility of such a system is demonstrated in these results which show that Rak expression can be roughly monitored by RT-PCR followed with electrochemical detection. This approach is rapid, in that electrochemical detection can be achieved in less than 1h following PCR. Comparable gel based methods routinely took over 2 h for running of the gel, fluorescent staining, scanning and image analysis. Gel based analysis is also limited by the number of potential samples that can be handled on a single gel. Electrochemical detection, on the other hand, is readily amenable to high though-put formulations. Ultimately, a hybridization type of electrochemical sensor using micron-sized electrodes could provide a rapid, inexpensive gene quantification system that could report actual mRNA quantities without *any* enzymatic manipulations.

REFERENCES:

1)Cance, W. G.; Craven, R. J.; Bergman, M.; Xu, L.; Alitalo, K.; Liu, E. T. *Cell Growth Dif.* **1994**, *5*, 1347-1355.

2)Craven, R. J.; Cance, W. G.; Liu, E. T. *Cancer Res.* **1995**, *55*, 3969-3972.

3)Armistead, P. M.; Thorp, H. H. *Analytical Chemistry in press* **2000**.

4)Johnston, D. H.; Glasgow, K. C.; Thorp, H. H. *J. Am. Chem. Soc.* **1995**, *117*, 8933-8938.

5)Revillion, F.; Hornez, L.; Peyrat, J.-P. *Clin. Chem.* **1997**, *43*, 2114-2120.

6)Totze, G.; Sachinidis, A.; Vettre, H.; Ko, Y. *Molecular and Cellular Probes* **1996**, *10*, 427-433.

7)Freeman, W. M.; Walker, S. J.; Vrana, K. E. *Biotechniques* **1999**, *26*, 112-125.

8)Willit, J. L.; Bowden, E. F. *J. Phys. Chem.* **1990**, *94*, 8241-8246.

9)Armistead, P. M.; Thorp, H. H. *Submitted to The Journal of the American Chemical Society* **2000**.

10)Armistead, P. M.; Thorp, H. H. *manuscript in preparation* **2000**.

11)Schneenberger, C.; Eder, S.; Speiser, P.; Zeillinger, R. *Anticancer Res.* **1996**, *16*, 849-852.

12)Schena, M.; Shalon, D.; Davis, R. W.; Brown, P. O. *Science* **1995**, *270*, 467-470.

13)Chee, M.; Yang, R.; Hubbell, E.; Berno, A.; Huang, X. C.; Stern, D.; Winkler, J.; Lockhart, D. J.; Morris, M. S.; Fodor, S. P. A. *Science* **1996**, *274*, 610-614.

14)DeRisi, J. L.; Iyer, V. R.; Brown, P. O. *Science* **1997**, *278*, 680-686.

15)Gray, N. S.; Wodicka, L.; Thunnissen, A.-M. W. H.; Norman, T. C.; Kwon, S.; Espinoza, F. H.; Morgan, D. O.; Barnes, G.; LeClerc, S.; Meijer, L.; Kim, S.-H.; Lockhart, D. J.; Schultz, P. G. *Science* **1998**, *281*, 533-538.

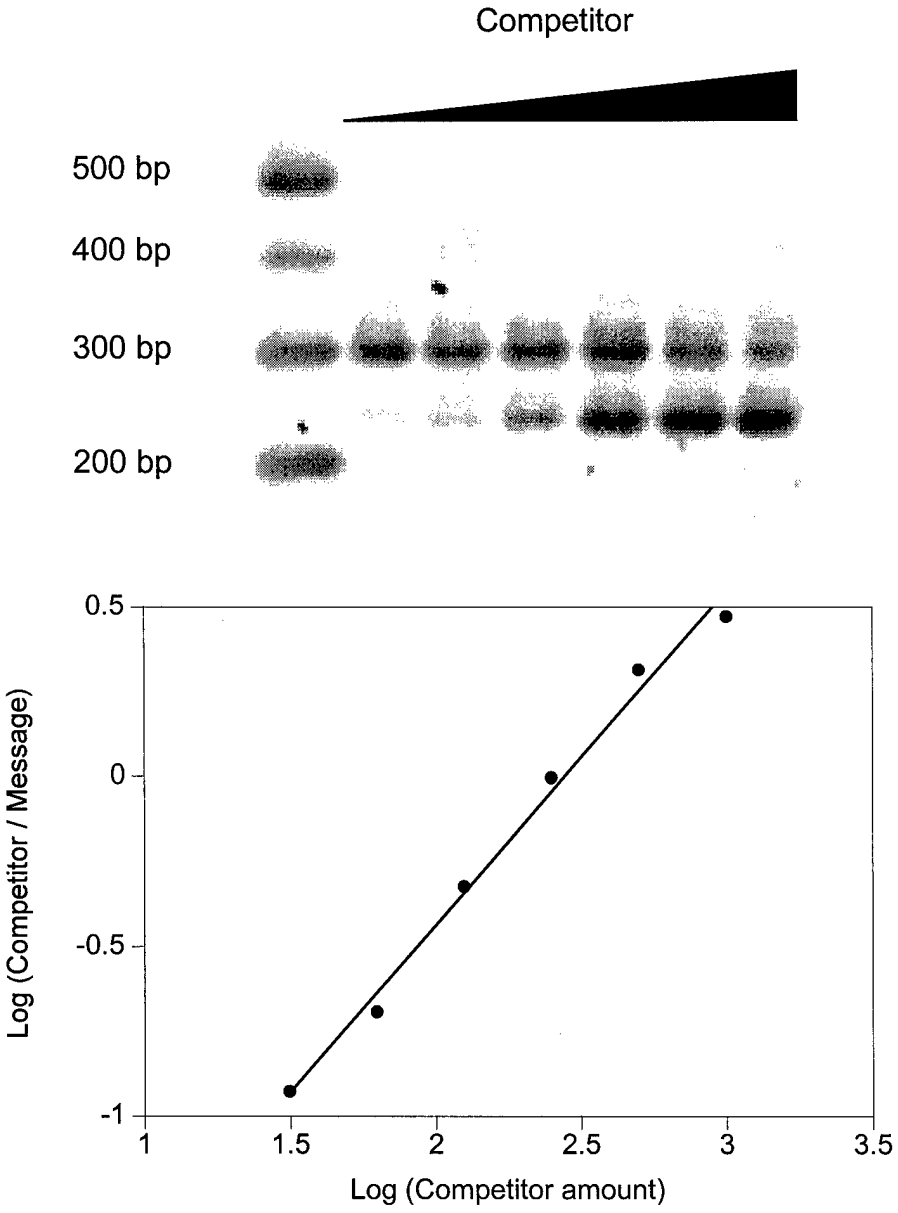

Figure 1: Competitive RT-PCR of Rak mRNA. Competitive RT-PCR was performed on RNA from sample 35B. As the amount of competitor added to each reaction increases, the ratio of the amount of native RT-PCR product (296 bp) decreases relative to the amount of competitor RT-PCR product (232 bp). The logarithm of the band intensity ratio versus the logarithm of the competitor amount is shown.

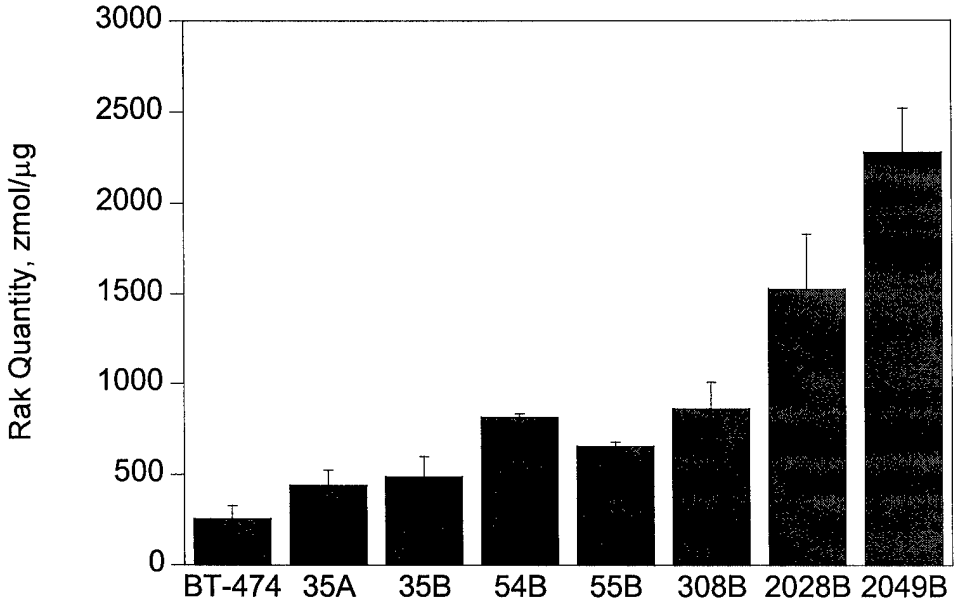

Figure 2: Combined Competitive RT-PCR Data. Rak mRNA quantity is given as zmol of Rak mRNA per µg of total RNA for the specified sample. Error bars show the standard deviation over 2 measurements.

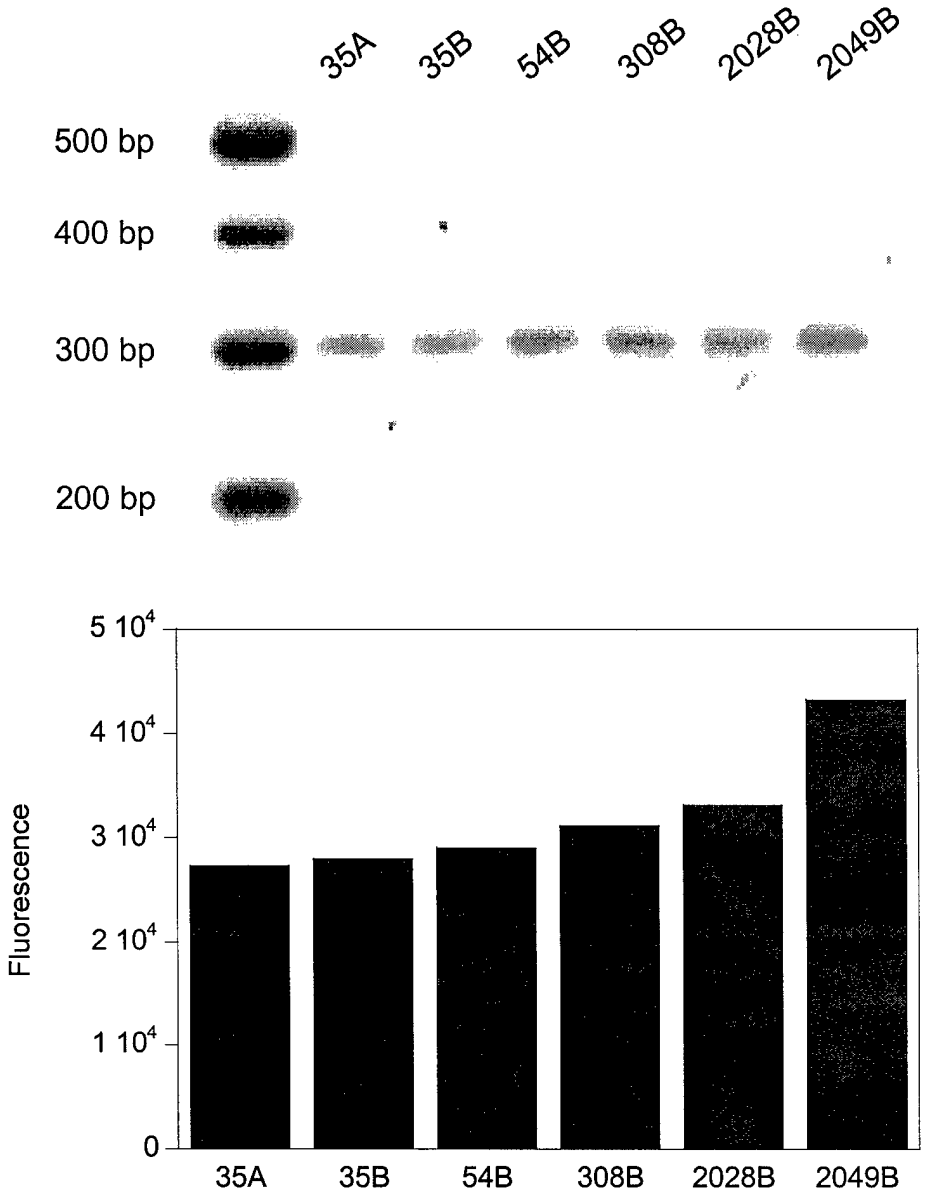

Figure 3: Gel Analysis of Rak RT-PCR Products before Purification. Fluorescence measurements for conventional RT-PCR products for each of six breast tissue samples were calculated using ImageQuaNT. Fluorescence intensities are not background subtracted.

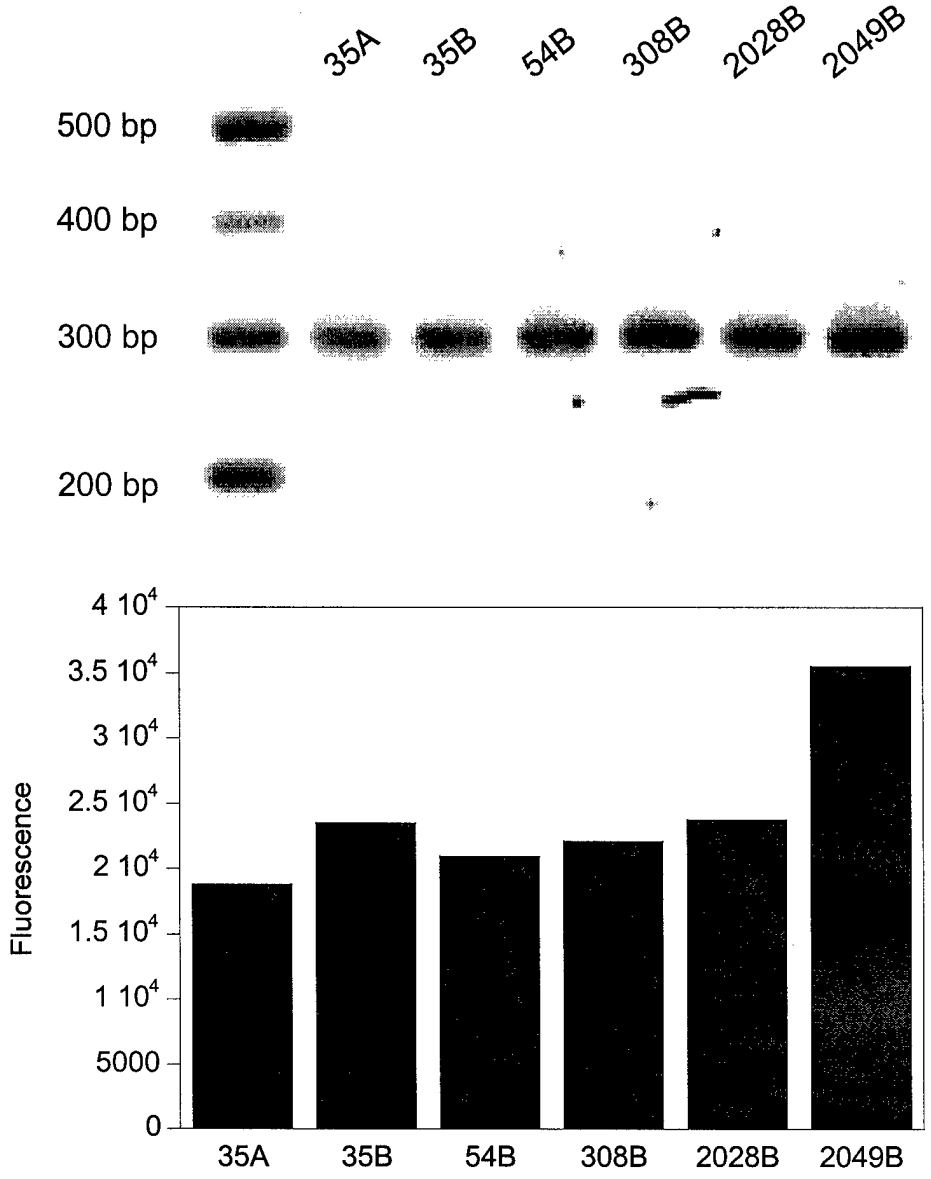

Figure 4: Gel Analysis of Rak RT-PCR Products after Purification. Fluorescence measurements for purified RT-PCR products for each of six breast tissue samples were calculated using ImageQuaNT. Fluorescence intensities are not background subtracted.

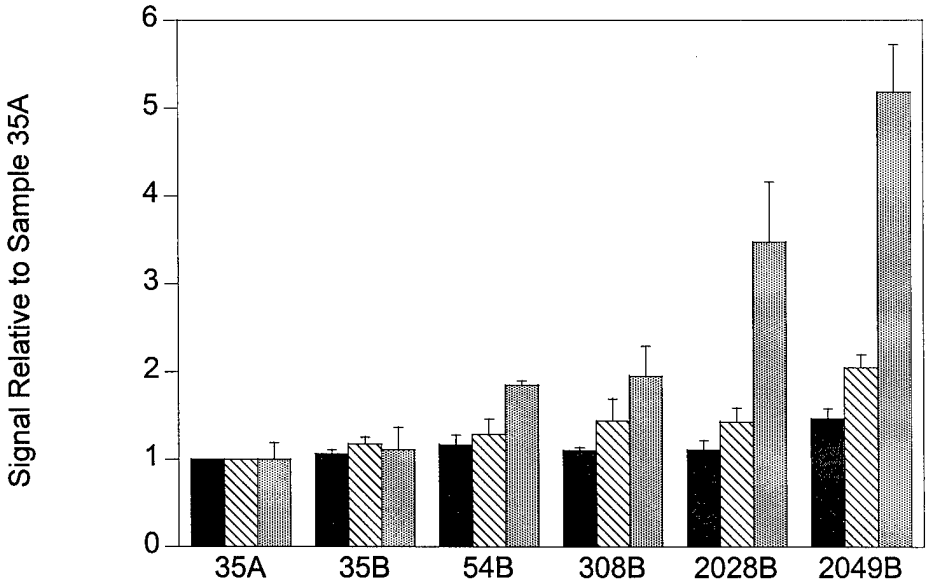

Figure 5: Signal Intensities for Rak RT-PCR and Competitive RT-PCR. The dark gray bars represent normalized gel band intensities for conventional RT-PCR of six breast tissue samples before purification. The striped bars represent the normalized band intensities for the same RT-PCR reactions after purification. The light gray bars represent the results from the competitive RT-PCR experiments that have been normalized to sample 35A. Error bars show the standard deviation over 2 measurements.

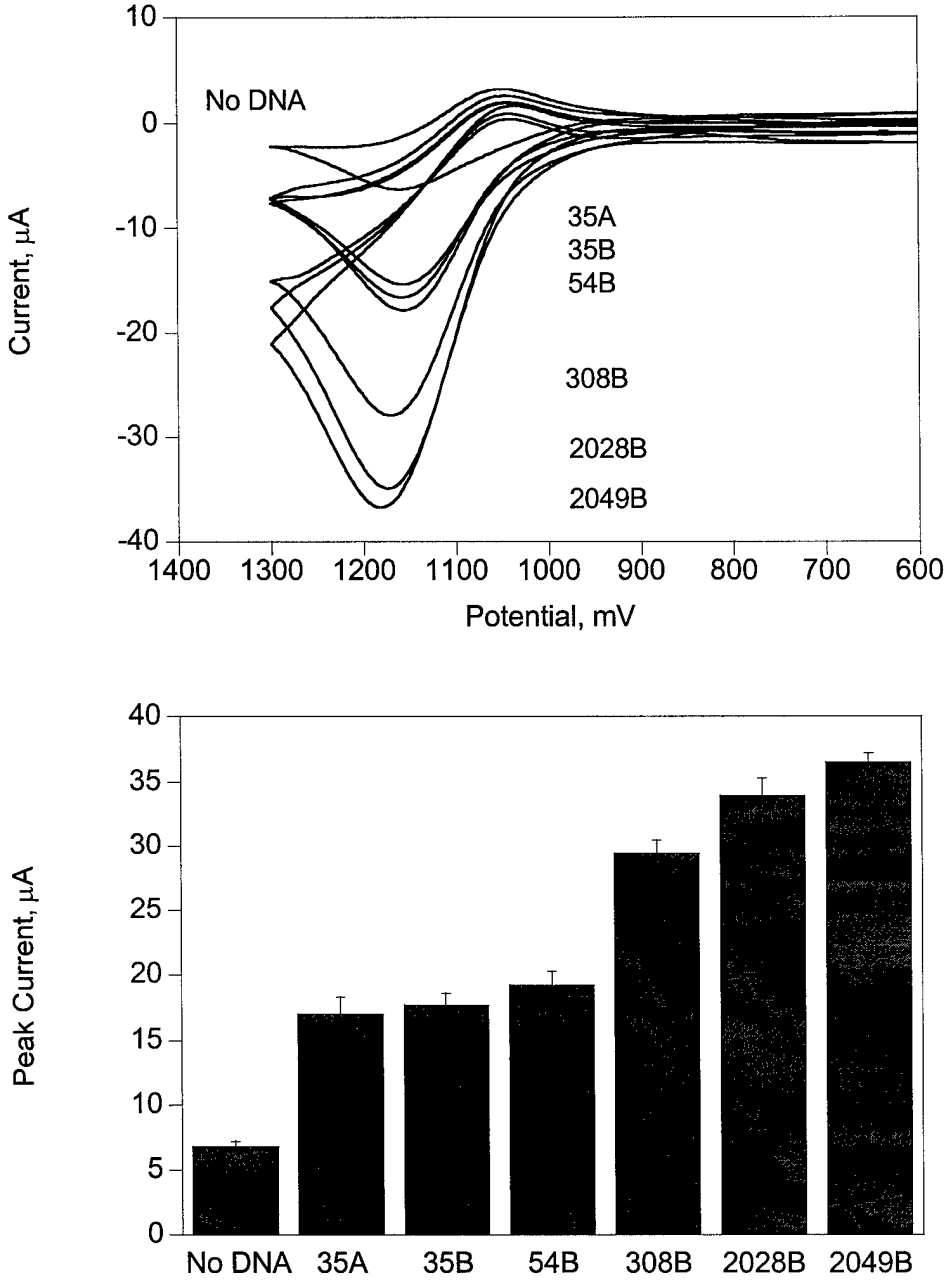

Figure 6: Cyclic Voltammograms of Rak RT-PCR Product-Modified Electrodes. Cyclic voltammograms for ITO electrodes modified by the RT-PCR products from six breast tissue samples are shown. The peak current for each sample is shown on the bar graph below.

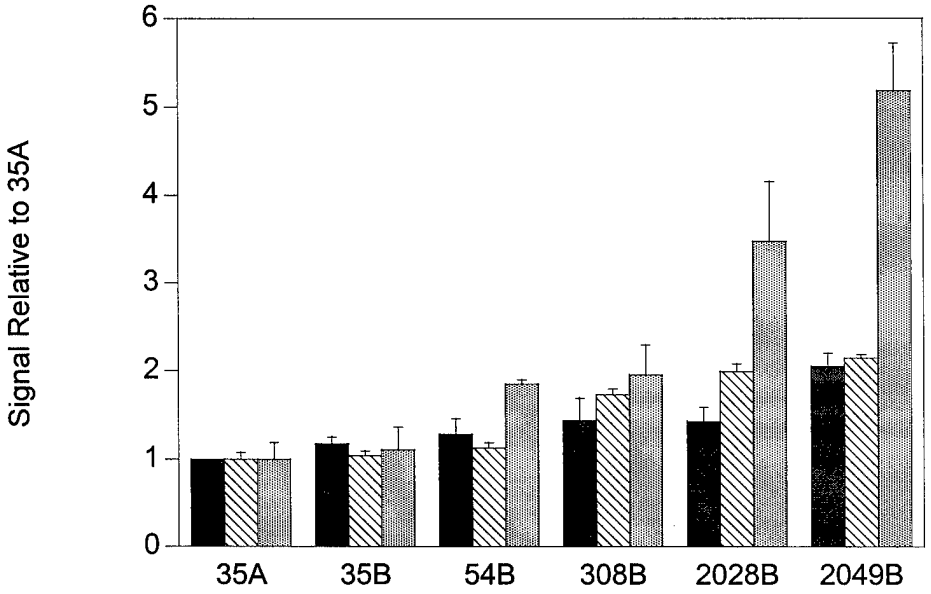

Figure 7: Comparison of Conventional and Electrochemical Quantification. The dark gray bars represent the post purification gel band intensities for the conventional RT-PCR experiments. The striped bars represent the cyclic voltammetry peak currents from the same conventional RT-PCR experiments. The light gray bars represent the competitive RT-PCR results. All signals are normalized to sample 35A.

PUBLICATIONS SUPPORTED BY THIS RESEARCH

Armistead, P. M., and Thorp, H. H. (2000). Measuring Rak mRNA Expression by RT-PCR with Catalytic Electrochemical Detection. in preparation.

Armistead, P. M., and Thorp, H. H. (2000). Modification of Metal Oxides with Nucleic Acids: Detection of Attomole Quantities of Immobilized DNA by Electrocatalysis. Analytical Chemistry *in press*.

MEETING ABSTRACTS

Abstract from the National M.D./Ph.D. Conference in Aspen, Colorado July 16-18 1999.

Electrochemical Detection of Femtomole Quantities of Surface Immobilized DNA
ARMISTEAD, P.*, THORP, H.
Department of Chemistry, University of North Carolina at Chapel Hill

The oxidation of guanine in DNA and RNA has been proposed as a possible method for the detection and quantification of clinically important nucleic acid sequences. Because of the slow electron transfer kinetics between guanine and most electrode materials an oxidation catalyst, $Ru(bpy)_3Cl_2$ (bpy = 2,2' bipyridyl), has been used to facilitate transfer of electrons from guanine to the electrode. This catalytic electrochemistry has been employed in the detection of several RT-PCR products that have been immobilized on a tin doped indium oxide (ITO) electrode.

HER-2 RT-PCR products were immobilized onto ITO electrodes. The electrodes were immersed in a solution containing 10 μM $Ru(bpy)_3^{2+}$ and cyclic voltammetry was performed from 0-1300 mV at a scan rate of 10 V/s. Experiments involving radiolabeled RT-PCR products revealed that this catalytic system could detect less than 1 fmol (1 fmol = 1×10^{-15} mol) of PCR product on a 10 mm^2 electrode and could discriminate a 50 fold difference in DNA quantity. The stability of the immobilized DNA on ITO has led us to investigate whether this system could be used as a hybridization sensor to detect either single-stranded cDNA or mRNA. Early experiments indicate that hybridization can be achieved; however, problems concerning non-specific attachment of the target to the electrode and poor sequence recognition need to be overcome.

For catalytic electrochemistry to become a viable technique for the detection of nucleic acid sequences the sensitivity of the system needs to improve. A system that used microelectrodes instead of the macroelectrodes used here should increase the technique's sensitivity by three orders of magnitude. At this level of sensitivity, quantification of clinically significant mRNAs, such as HER-2, could be accomplished from small tissue samples without the need for amplification.

Abstract from the Department of Defense Era of Hope Conference in Atlanta GA, June 8-12, 2000.

Monitoring Rak Expression through Electrocatalysis
ARMISTEAD, P.*, THORP, H.

Department of Chemistry, University of North Carolina at Chapel Hill

The use of small molecules to inhibit overexpressed signaling molecules in various tumors is a general approach in the treatment of cancer. Consequently, there is a need to develop rapid sensors that can monitor gene expression either at the RNA or the protein level. The purpose of this study was to develop an electrochemical method to monitor expression of the nuclear tyrosine kinase, Rak, first in cultured cell lines and then in breast tissue samples.

Rak expression levels were first measured in BT-474 cells through the use of competitive RT-PCR. Competitive RT-PCR is an extremely sensitive technique that can provide absolute quantification of any specific mRNA in a total cellular extract. These experiments established Rak expression to be 120 zmol (1 zmol = $1E^{-21}$ mol) per μg of BT-474 RNA.

Having established this low level of expression in this model cell line, parallel RT-PCR was performed on RNA extracted from breast tissue samples. Differences in Rak expression were monitored in two ways. Agarose gel electrophoresis was performed as a traditional assay. The second method involved attaching the PCR product to an indium tin oxide (ITO) electrode and detecting its oxidation in the presence of an electron transfer catalyst, $Ru(bpy)_3^{2+}$ (bpy = 2,2' bipyridine).

The development of a competitive RT-PCR assay for Rak expression will allow accurate determination of Rak expression in many different tissue types. The electrochemical method developed in this project provides a rapid and sensitive way to monitor gene expression from multiple tissue samples at once.

PERSONNEL RECEIVING PAY FROM THIS RESEARCH

Paul Armistead.